

P-GLYCOPROTEINS AND USES THEREOF

Related Applications

This application is a divisional of US patent application serial number 09/672,810,
5 filed September 28, 2000, now pending, which application claims priority under 35 U.S.C.
§119 to U.S. provisional application serial number 60/158,818, filed October 12, 1999, and to
U.S. provisional application serial number 60/156,921, filed September 28, 1999.

Field of the Invention

10 The invention pertains to P-glycoproteins of cynomolgous monkey (*Macaca*
fascicularis).

Background of the Invention

P-glycoprotein (PGP; also known as multidrug transporter, MDR1) is a member of the
15 ABC transporter superfamily and is expressed in the human intestine, liver and other tissues.
This enzyme serves as an efflux pump exporting small molecules across the cell membrane.
It has been known for several years that high level expression of PGP is a mechanism for
tumor resistance to cancer chemotherapy. Intestinal expression of PGP may affect the oral
20 bioavailability of drug molecules that are substrates for this transporter. PGP can efficiently
efflux drugs back into the intestinal lumen and thus reduce the amount of drug that enters into
circulation.

The measurement of interaction with PGP can provide a better understanding of the
reasons why particular drugs demonstrate low or high bioavailability. Interaction with PGP
can be studied using either direct assays of drug transport in polarized cell systems or with
25 indirect assays such as drug-stimulated ATPase activity and inhibition of the transport of
fluorescent substrates.

Therefore there is a need for additional PGP polypeptides, preferably which are
closely related to the human PGP, for use in the foregoing drug assays.

Summary of the Invention

30 Nucleic acids encoding the P-glycoprotein of cynomolgous monkey (*Macaca*
fascicularis) have now been identified, isolated, cloned and sequenced. This PGP is closely

related (has a high degree of identity) to the human PGP. The invention provides isolated nucleic acid molecules, unique fragments of those molecules, expression vectors containing the foregoing, and host cells transfected with those molecules. The invention also provides isolated polypeptides and inhibitors of the foregoing nucleic acids and polypeptides which reduce drug transport. The PGP nucleic acids and polypeptides are useful in assays for evaluating bioavailability of drugs, as well as for the optimization or discovery of drugs. In addition, the foregoing can be used in the diagnosis or treatment of conditions characterized by PGP activity and can be used in methods in which it is therapeutically useful to increase or decrease PGP activity.

According to one aspect of the invention, isolated nucleic acid molecules are provided selected from the group consisting of (a) nucleic acid molecules that code for the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4, (b) allelic variants of (a), and (c) complements of (a) or (b). In certain embodiments, the isolated nucleic acid molecule codes for SEQ ID NO:2 or SEQ ID NO:4. In other embodiments, the isolated nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO:2 or SEQ ID NO:4.

According to another aspect of the invention, isolated P-glycoprotein polypeptides or fragments thereof are provided which include at least one amino acid of a cynomolgous P-glycoprotein selected from the group consisting of amino acids 12, 24, 30, 74, 78, 86, 89, 90, 91, 92, 95, 97, 99, 102, 103, 104, 185, 324, 363, 518, 635, 650, 656, 659, 677, 730, 738, 742, 745, 761, 765, 835, 851, 921, 967, 1003, 1027, 1038, 1048, 1103, 1128, 1168 and 1277 of SEQ ID NO:2 and amino acids 93, 94 and 95 of SEQ ID NO:4, wherein the P-glycoprotein is identical to a human P-glycoprotein except for the at least one amino acid of a cynomolgous P-glycoprotein. In certain embodiments, the human P-glycoprotein is selected from the group of SEQ ID NO:5 and SEQ ID NO:6.

According to yet another aspect of the invention, isolated P-glycoprotein polypeptides or fragments thereof which include at least one amino acid of a cynomolgous P-glycoprotein selected from the group consisting of amino acids 3, 6, 8, 10, 13, 17, 19, 20, 21, 26, 30, 36, 38, 48, 52, 56, 64, 74, 78, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 98, 100, 101, 102, 103, 104, 105, 106, 110, 113, 145, 190, 197, 210, 231, 319, 324, 327, 345, 363, 395, 451, 455, 456, 468, 473, 494, 518, 530, 631, 641, 642, 648, 650, 655, 656, 664, 665, 672, 673, 674, 675, 683, 687, 689, 691, 692, 694, 701, 705, 715, 729, 730, 734, 742, 743, 745, 754, 757, 765, 835, 912, 918, 921, 940, 941, 944, 966, 967, 968, 970, 972, 981, 1008, 1015, 1023,

1024, 1048, 1093, 1096, 1103, 1128, 1142, 1146, 1147, 1156, 1160, 1163, 1166, 1250 and
1271 of SEQ ID NO:2 and amino acids 93 and 94 of SEQ ID NO:4, wherein the P-
glycoprotein is identical to a dog P-glycoprotein except for the at least one amino acid of a
cynomologous P-glycoprotein. In some embodiments, the dog P-glycoprotein is selected
5 from the group of SEQ ID NO:7 and SEQ ID NO:8.

In preferred embodiments, the isolated P-glycoprotein polypeptides or fragments
thereof include an amino acid sequence selected from the group consisting of SEQ ID NO:2,
fragments of SEQ ID NO:2, SEQ ID NO:4 and fragments of SEQ ID NO:4.. Yet other
polypeptides include combinations of the foregoing dog, human and cynomologous PGP
10 polypeptides.

According to still other embodiments of the invention, isolated nucleic acid molecules
are provided which encode the foregoing isolated P-glycoprotein polypeptides or fragments
thereof. Also included expression vectors comprising the foregoing isolated nucleic acid
molecules operably linked to a promoter, as well as host cells transformed or transfected with
15 the expression vectors.

In another aspect of the invention, agents which selectively binds the isolated PGP
polypeptides are provided. Preferably the agent does not bind a human or dog P-glycoprotein,
except those provided herein. In certain embodiments, the agent is a polypeptide preferably
one selected from the group consisting of monoclonal antibodies, polyclonal antibodies, Fab
20 antibody fragments, F(ab)₂ antibody fragments and antibody fragments including a CDR3
region. Also provided are agents which selectively binds the foregoing isolated nucleic acid
molecules, preferably antisense nucleic acid molecules which selectively binds to the isolated
nucleic acid molecule.

According to another aspect of the invention, methods for predicting the
25 bioavailability of a compound are provided. The methods include measuring the
transmembrane transport of a test compound by a first P-glycoprotein, comparing the
transmembrane transport of the test compound by the first P-glycoprotein and a second
P-glycoprotein to predict the bioavailability of the test compound, wherein the relative
amount or rate of transport by the first P-glycoprotein and the second P-glycoprotein is
30 predictive of bioavailability of the test compound. In certain embodiments the first P-
glycoprotein is selected from the group consisting of dog P-glycoproteins and primate P-
glycoproteins, preferably one of the foregoing polypeptides. In other embodiments the

second P-glycoprotein is a human P-glycoprotein.

In still other aspects of the invention, methods for inhibiting P-glycoprotein transporter activity in a mammalian cell are provided. The methods include contacting the mammalian cell with an amount of one of the foregoing agents effective to inhibit
5 P-glycoprotein transporter activity in the mammalian cell.

Also included in the invention are methods for increasing bioavailability of a drug in a subject. The methods include administering to a subject in need of such treatment one of the foregoing agents in an amount effective to increasing bioavailability of a drug. The inhibitor can be administered prior to administering the drug, or concurrently with the drug.

10 Also provided are methods for increasing P-glycoprotein transporter activity in a cell. These methods include contacting the cell with a molecule selected from the group consisting of the foregoing nucleic acid molecules, in an amount effective to increase P-glycoprotein transporter activity in the cell. The cell can be contacted under conditions whereby the P-glycoprotein is expressed.

15 According to yet another aspect of the invention, methods for identifying lead compounds for a pharmacological agent useful in the treatment of disease associated with P-glycoprotein transporter activity are provided. The methods include providing a cell or other membrane-encapsulated space comprising a P-glycoprotein as provided herein; contacting the cell or other membrane-encapsulated space with a candidate pharmacological
20 agent under conditions which, in the absence of the candidate pharmacological agent, cause a first amount of P-glycoprotein transporter activity; and determining a second amount of P-glycoprotein transporter activity as a measure of the effect of the pharmacological agent on the P-glycoprotein transporter activity, wherein a second amount of P-glycoprotein transporter activity which is less than the first amount indicates that the candidate
25 pharmacological agent is a lead compound for a pharmacological agent which reduces P-glycoprotein transporter activity and wherein a second amount of P-glycoprotein transporter activity which is greater than the first amount indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which increases P-glycoprotein transporter activity. The methods can further include a step of loading the cell or other
30 membrane-encapsulated space with a detectable compound, wherein the compound is detected as a measure of the P-glycoprotein transporter activity.

Also included are methods for identifying compounds which selectively bind a

P-glycoprotein. The methods include contacting a P-glycoprotein provided herein with a compound, and determining the binding of the compound to the P-glycoprotein. The methods can further include determining the effect of the compound on the P-glycoprotein transporter activity of the P-glycoprotein or determining the effect of the compound on the ATPase activity of the P-glycoprotein.

5 Additional methods provided according to the invention include methods for determining ATPase activity of a P-glycoprotein. The methods include contacting a host cell as provided above, or a membrane fraction thereof, with a test drug, and measuring ATPase activity of the P-glycoprotein. In certain embodiments, the step of measuring ATPase activity 10 is performed at least twice at different times. Also provided methods for determining transmembrane transport of a compound by a P-glycoprotein. The methods include contacting a host cell provided above, or a membrane fraction thereof, with a test drug, and measuring transport of the test drug under sink conditions in at least one direction of transport selected from the group consisting of the apical to basolateral direction and the basolateral to 15 apical direction. In certain embodiments the step of measuring transport of the test drug is performed at least twice at different times.

These and other aspects of the invention are described in greater detail below.

Brief Description of the Sequences

20 SEQ ID NO:1 is the nucleotide sequence encoding cynomolgous monkey P-glycoprotein.

SEQ ID NO:2 is the amino acid sequence of a cynomolgous monkey P-glycoprotein encoded by SEQ ID NO:1.

25 SEQ ID NO:3 is the nucleotide sequence of a cynomolgous monkey P-glycoprotein allele having a 9 nucleotide insert relative to SEQ ID NO:1.

SEQ ID NO:4 is the amino acid sequence of a cynomolgous monkey P-glycoprotein allelic variant encoded by SEQ ID NO:3, having a 3 amino acid insert.

SEQ ID NO:5 is the amino acid sequence of a human P-glycoprotein having Genbank accession number M14758.

30 SEQ ID NO:6 is the amino acid sequence of a human P-glycoprotein having Genbank accession numbers AF016535 or NM_000927.

SEQ ID NO:7 is the amino acid sequence of a dog P-glycoprotein having Genbank

accession number AF045016.

SEQ ID NO:8 is the amino acid sequence of a dog P-glycoprotein having Genbank accession numbers AF092810.

SEQ ID NO:9 is the nucleotide sequence of a primer based on the human PGP
5 nucleotide sequence.

SEQ ID NO:10 is the nucleotide sequence of a primer based on the human PGP
nucleotide sequence.

SEQ ID NO:11 is the nucleotide sequence of a primer based on the human PGP
nucleotide sequence.

10 SEQ ID NO:12 is the nucleotide sequence of a primer based on the cynomologous
PGP nucleotide sequence.

SEQ ID NO:13 is the nucleotide sequence of a primer based on the cynomologous
PGP nucleotide sequence.

15 SEQ ID NO:14 is the nucleotide sequence of a primer based on the T7 promoter
nucleotide sequence.

SEQ ID NO:15 is the nucleotide sequence of a primer based on the cynomologous and
human PGP nucleotide sequences.

SEQ ID NO:16 is the nucleotide sequence of a primer based on the cynomologous and
human PGP nucleotide sequences.

20 SEQ ID NO:17 is the nucleotide sequence of a primer based on the cynomologous
PGP nucleotide sequence.

SEQ ID NO:18 is the nucleotide sequence of a primer based on the cynomologous
PGP nucleotide sequence.

25 **Detailed Description of the Invention**

The present invention in one aspect involves the identification of cDNAs encoding
cynomologous monkey P-glycoproteins, referred to herein as cynomologous PGP. The
nucleotide sequences of the cynomologous PGP are presented as SEQ ID Nos:1 and 3, and
the amino acid sequences of the cynomologous PGP are presented as SEQ ID Nos:2 and 4.

30 The nucleotide and amino acid sequences of a cynomologous PGP allelic variant (SEQ ID
NOS:3 and 4) have inserts of 9 nucleotides and 3 amino acids, respectively, but are otherwise
identical to SEQ ID NOS:1 and 2. The closely related human PGP was deposited in

GenBank under accession number M14758. Whereas much of the polypeptides presented herein is identical to human PGP, cynomologous PGP has several single amino acid differences and a N-terminal domain of about 19-34 amino acids that is about 36-58% identical to human PGP. The insert present in the allelic variant referred to above (SEQ ID 5 NOS:3 and 4) is located near the end of this cynomologous-specific domain. Surprisingly, the N-terminal domain of the cynomologous PGP that differs from the human amino acid sequence is located in a portion of the molecule in which the cynomologous and human amino acid sequences are otherwise 100% identical. This species difference in the very highly conserved protein domains of the P-glycoprotein is entirely unexpected.

10 The invention involves in one aspect cynomologous PGP nucleic acids and polypeptides, as well as therapeutics relating thereto. The invention also embraces isolated functionally equivalent variants, useful analogs and fragments of the foregoing nucleic acids and polypeptides; complements of the foregoing nucleic acids; and molecules which selectively bind the foregoing nucleic acids and polypeptides.

15 The cynomologous PGP nucleic acids and polypeptides of the invention are isolated. As used herein with respect to nucleic acids, the term “isolated” means: (i) amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii) recombinantly produced by cloning; (iii) purified, as by cleavage and gel separation; or (iv) synthesized by, for example, chemical synthesis. An isolated nucleic acid is one which is readily manipulable by recombinant DNA 20 techniques well known in the art. Thus, a nucleotide sequence contained in a vector in which 5' and 3' restriction sites are known or for which polymerase chain reaction (PCR) primer sequences have been disclosed is considered isolated but a nucleic acid sequence existing in its native state in its natural host is not. An isolated nucleic acid may be substantially purified, but need not be. For example, a nucleic acid that is isolated within a cloning or 25 expression vector is not pure in that it may comprise only a tiny percentage of the material in the cell in which it resides. Such a nucleic acid is isolated, however, as the term is used herein because it is readily manipulable by standard techniques known to those of ordinary skill in the art. An isolated nucleic acid as used herein is not a naturally occurring chromosome.

30 As used herein with respect to polypeptides, “isolated” means separated from its native environment and present in sufficient quantity to permit its identification or use. Isolated, when referring to a protein or polypeptide, means, for example: (i) selectively

produced by expression cloning or (ii) purified as by chromatography or electrophoresis. Isolated proteins or polypeptides may be, but need not be, substantially pure. The term "substantially pure" means that the proteins or polypeptides are essentially free of other substances with which they may be found in nature or *in vivo* systems to an extent practical
5 and appropriate for their intended use. Substantially pure polypeptides may be produced by techniques well known in the art. Because an isolated protein may be admixed with a pharmaceutically acceptable carrier in a pharmaceutical preparation, the protein may comprise only a small percentage by weight of the preparation. The protein is nonetheless isolated in that it has been separated from the substances with which it may be associated in
10 living systems, i.e. isolated from other proteins.

As used herein a cynomolgous PGP nucleic acid refers to an isolated nucleic acid molecule which codes for a cynomolgous PGP polypeptide. Such nucleic acid molecules code for cynomolgous PGP polypeptides which include the sequence of SEQ ID NOs:2 and 4, and fragments thereof. The nucleic acid molecules include the nucleotide sequences of
15 SEQ ID Nos:1 and 3, and nucleotide sequences which differ from the sequences of SEQ ID NOs:1 and 3 in codon sequence due to the degeneracy of the genetic code. The cynomolgous PGP nucleic acids of the invention also include alleles of the foregoing nucleic acids, as well as fragments of the foregoing nucleic acids. Such fragments can be used, for example, as probes in hybridization assays and as primers in a polymerase chain reaction
20 (PCR). Preferred cynomolgous PGP nucleic acids include the nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:3. Complements of the foregoing nucleic acids also are embraced by the invention.

As used herein "cynomolgous PGP activity" refers to an ability of a PGP polypeptide to export small molecules across the cell membrane. A molecule which inhibits
25 cynomolgous PGP activity (an antagonist) is one which inhibits export of small molecules via PGP and a molecule which increases cynomolgous PGP activity (an agonist) is one which increases export of small molecules via PGP. Changes in cynomolgous PGP activity can be measured by assays such as those disclosed herein, including efflux of fluorescent compounds from cells.

Alleles of the cynomolgous PGP nucleic acids of the invention can be identified by conventional techniques. For example, alleles of cynomolgous PGP can be isolated by hybridizing a probe which includes at least a fragment of SEQ ID NO:1 or SEQ ID NO:3
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under stringent conditions with a cDNA library and selecting positive clones. Thus, an aspect of the invention is those nucleic acid sequences which code for cynomolgous PGP polypeptides and which hybridize to a nucleic acid molecule consisting of SEQ ID NO:1 or SEQ ID NO:3 under stringent conditions. The term "stringent conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. More specifically, stringent conditions, as used herein, refers, for example, to hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin, 2.5mM NaH₂PO₄(pH7), 0.5% SDS, 2mM EDTA). SSC is 0.15M sodium chloride/0.15M sodium citrate, pH7; SDS is sodium dodecyl sulphate; and EDTA is ethylenediaminetetraacetic acid. After hybridization, the membrane upon which the DNA is transferred is washed at 2 x SSC at room temperature and then at 0.1 - 0.5 x SSC/0.1 x SDS at temperatures up to 68°C.

There are other conditions, reagents, and so forth which can be used, which result in a similar degree of stringency. The skilled artisan will be familiar with such conditions, and thus they are not given here. It will be understood, however, that the skilled artisan will be able to manipulate the conditions in a manner to permit the clear identification of alleles of cynomolgous PGP nucleic acids of the invention. The skilled artisan also is familiar with the methodology for screening cells and libraries for expression of such molecules which then are routinely isolated, followed by isolation of the pertinent nucleic acid molecule and sequencing.

In screening for cynomolgous PGP nucleic acids, a Southern blot may be performed using the foregoing stringent conditions, together with a radioactive probe. After washing the membrane to which the DNA is finally transferred, the membrane can be placed against X-ray film to detect the radioactive signal.

The cynomolgous PGP nucleic acids of the invention also include degenerate nucleic acids which include alternative codons to those present in the native materials. For example, serine residues are encoded by the codons TCA, AGT, TCC, TCG, TCT and AGC. Each of the six codons is equivalent for the purposes of encoding a serine residue. Thus, it will be

apparent to one of ordinary skill in the art that any of the serine-encoding nucleotide triplets may be employed to direct the protein synthesis apparatus, *in vitro* or *in vivo*, to incorporate a serine residue into an elongating cynomolgous PGP polypeptide. Similarly, nucleotide sequence triplets which encode other amino acid residues include, but are not limited to:

5 CCA, CCC, CCG and CCT (proline codons); CGA, CGC, CGG, CGT, AGA and AGG (arginine codons); ACA, ACC, ACG and ACT (threonine codons); AAC and AAT (asparagine codons); and ATA, ATC and ATT (isoleucine codons). Other amino acid residues may be encoded similarly by multiple nucleotide sequences. Thus, the invention embraces degenerate nucleic acids that differ from the biologically isolated nucleic acids in

10 codon sequence due to the degeneracy of the genetic code.

The invention also provides modified nucleic acid molecules which include additions, substitutions and deletions of one or more nucleotides. In preferred embodiments, these modified nucleic acid molecules and/or the polypeptides they encode retain at least one activity or function of the unmodified nucleic acid molecule and/or the polypeptides, such as

15 transporter activity, etc. In certain embodiments, the modified nucleic acid molecules encode modified polypeptides, preferably polypeptides having conservative amino acid substitutions as are described elsewhere herein. The modified nucleic acid molecules are structurally related to the unmodified nucleic acid molecules and in preferred embodiments are sufficiently structurally related to the unmodified nucleic acid molecules so that the modified

20 and unmodified nucleic acid molecules hybridize under stringent conditions known to one of skill in the art.

For example, modified nucleic acid molecules which encode polypeptides having single amino acid changes can be prepared. Each of these nucleic acid molecules can have one, two or three nucleotide substitutions exclusive of nucleotide changes corresponding to

25 the degeneracy of the genetic code as described herein. Likewise, modified nucleic acid molecules which encode polypeptides having two amino acid changes can be prepared which have, e.g., 2-6 nucleotide changes. Numerous modified nucleic acid molecules like these will be readily envisioned by one of skill in the art, including for example, substitutions of nucleotides in codons encoding amino acids 2 and 3, 2 and 4, 2 and 5, 2 and 6, and so on. In

30 the foregoing example, each combination of two amino acids is included in the set of modified nucleic acid molecules, as well as all nucleotide substitutions which code for the amino acid substitutions. Additional nucleic acid molecules that encode polypeptides having

additional substitutions (i.e., 3 or more), additions or deletions (e.g., by introduction of a stop codon or a splice site(s)) also can be prepared and are embraced by the invention as readily envisioned by one of ordinary skill in the art. Any of the foregoing nucleic acids or polypeptides can be tested by routine experimentation for retention of structural relation or 5 activity to the nucleic acids and/or polypeptides disclosed herein.

The invention also provides isolated fragments of SEQ ID NO:1 and SEQ ID NO:3. The fragments can be used as probes in Southern blot assays to identify such nucleic acids, or can be used in amplification assays such as those employing PCR. Smaller fragments are those comprising 12, 13, 14, 15, 16, 17, 18, 20, 22, 25, 30, 40, 50, or 75 nucleotides, and 10 every integer therebetween, and are useful e.g. as primers for nucleic acid amplification procedures. As known to those skilled in the art, larger probes such as 200, 250, 300, 400 or more nucleotides are preferred for certain uses such as Southern blots, while smaller fragments will be preferred for uses such as PCR. Fragments also can be used to produce fusion proteins for generating antibodies or determining binding of the polypeptide fragments. 15 Likewise, fragments can be employed to produce non-fused fragments of the cynomologous PGP polypeptides, useful, for example, in the preparation of antibodies, in immunoassays, and the like. The foregoing nucleic acid fragments further can be used as antisense molecules to inhibit the expression of cynomologous PGP nucleic acids and polypeptides, particularly for therapeutic purposes as described in greater detail below.

20 The invention also includes functionally equivalent variants of the cynomologous PGP, which include variant nucleic acids and polypeptides which retain one or more of the functional properties of the cynomologous PGP. Preferably such variants include the cynomologous-specific N-terminal domain (e.g., amino acids 86 - 104 of SEQ ID NO:2 or amino acids 86 - 101 of SEQ ID NO:4). For example, variants include a fusion protein which 25 includes the extracellular and transmembrane domains of the cynomologous PGP which retains the ability to transport molecules. Still other functionally equivalent variants include truncations, deletions, point mutations, or additions of amino acids to the sequence of SEQ ID NOs:2 or 4 which retain functions of SEQ ID NOs:2 or 4. Functionally equivalent variants also include a cynomologous PGP which has had a portion of the N-terminus removed or 30 replaced by a similar domain from another P-glycoprotein (e.g. a "domain-swapping" variant). Other functionally equivalent variants will be known to one of ordinary skill in the art, as will methods for preparing such variants. The activity of a functionally equivalent

variant can be determined using the methods provided herein, and in references that have described assays using P-glycoproteins of other species. Such variants are useful, *inter alia*, for evaluating bioavailability of drugs, in assays for identification of compounds which bind and/or regulate the transporter function of the cynomolgous PGP, and for determining the portions of the cynomolgous PGP which are required for transporter activity.

5 Variants which are non-functional also can be prepared as described above. Such variants are useful, for example, as negative controls in experiments testing transporter activity.

A cynomolgous PGP nucleic acid, in one embodiment, is operably linked to a gene expression sequence which directs the expression of the cynomolgous PGP nucleic acid within a eukaryotic or prokaryotic cell. The “gene expression sequence” is any regulatory nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the cynomolgous PGP nucleic acid to which it is operably linked. The gene expression sequence may, for example, be a 10 mammalian or viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPTR), adenosine deaminase, pyruvate kinase, β -actin promoter and other constitutive promoters. Exemplary viral promoters which function constitutively in eukaryotic cells include, for example, promoters from the simian virus, 15 papilloma virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus, the long terminal repeats (LTR) of Moloney murine leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other constitutive promoters are known to those of ordinary skill in the art. The promoters useful as 20 gene expression sequences of the invention also include inducible promoters. Inducible promoters are expressed in the presence of an inducing agent. For example, the metallothionein promoter is induced to promote transcription and translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the 25 art.

In general, the gene expression sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and 30 translation, respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing sequences will include a promoter region which

includes a promoter sequence for transcriptional control of the operably joined cynomologous PGP nucleic acid. The gene expression sequences optionally includes enhancer sequences or upstream activator sequences as desired.

The cynomologous PGP nucleic acid sequence and the gene expression sequence are
5 said to be “operably linked” when they are covalently linked in such a way as to place the transcription and/or translation of the cynomologous PGP coding sequence under the influence or control of the gene expression sequence. If it is desired that the cynomologous PGP sequence be translated into a functional protein, two DNA sequences are said to be operably linked if induction of a promoter in the 5' gene expression sequence results in the
10 transcription of the cynomologous PGP sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the cynomologous PGP sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence would be operably
15 linked to a cynomologous PGP nucleic acid sequence if the gene expression sequence were capable of effecting transcription of that cynomologous PGP nucleic acid sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The cynomologous PGP nucleic acid molecules and the cynomologous PGP polypeptides (including the cynomologous PGP inhibitors described below) of the invention
20 can be delivered to the eukaryotic or prokaryotic cell alone or in association with a vector. In its broadest sense, a “vector” is any vehicle capable of facilitating: (1) delivery of a cynomologous PGP nucleic acid or polypeptide to a target cell, (2) uptake of a cynomologous PGP nucleic acid or polypeptide by a target cell, or (3) expression of a cynomologous PGP nucleic acid molecule or polypeptide in a target cell. Preferably, the vectors transport the
25 cynomologous PGP nucleic acid or polypeptide into the target cell with reduced degradation relative to the extent of degradation that would result in the absence of the vector. Optionally, a “targeting ligand” can be attached to the vector to selectively deliver the vector to a cell which expresses on its surface the cognate receptor (e.g. a receptor, an antigen recognized by an antibody) for the targeting ligand. In this manner, the vector (containing a
30 cynomologous PGP nucleic acid or a cynomologous PGP polypeptide) can be selectively delivered to a specific cell. In general, the vectors useful in the invention are divided into two classes: biological vectors and chemical/physical vectors. Biological vectors are more useful

for delivery/uptake of cynomolgous PGP nucleic acids to/by a target cell. Chemical/physical vectors are more useful for delivery/uptake of cynomolgous PGP nucleic acids or cynomolgous PGP proteins to/by a target cell.

Biological vectors include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the nucleic acid sequences of the invention, and free nucleic acid fragments which can be linked to the nucleic acid sequences of the invention. Viral vectors are a preferred type of biological vector and include, but are not limited to, nucleic acid sequences from the following viruses: retroviruses, such as Moloney murine leukemia virus; Harvey murine sarcoma virus; murine mammary tumor virus; Rous sarcoma virus; adenovirus; adeno-associated virus; SV40-type viruses; polyoma viruses; poxviruses; retroviruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; and polio virus. One can readily employ other vectors not named but known in the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. In general, the retroviruses are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes *in vivo*. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, M., "Gene Transfer and Expression, A Laboratory Manual," W.H. Freeman C.O., New York (1990) and Murry, E.J. Ed. "Methods in Molecular Biology," vol. 7, Humana Press, Inc., Clifton, New Jersey (1991).

Another preferred virus for certain applications is the adeno-associated virus, a double-stranded DNA virus. The adeno-associated virus can be engineered to be replication-deficient and is capable of infecting a wide range of cell types and species. It further has advantages, such as heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages; and lack of superinfection inhibition thus allowing multiple series of

transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of 5 selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Expression vectors containing all the necessary elements for expression are commercially available and known to those skilled in the art. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory 10 Press, 1989. Cells are genetically engineered by the introduction into the cells of heterologous DNA (RNA) encoding a cynomolgous PGP polypeptide or fragment or variant thereof. That heterologous DNA (RNA) is placed under operable control of transcriptional elements to permit the expression of the heterologous DNA in the host cell.

Preferred systems for mRNA expression in mammalian cells are those such as 15 pRc/CMV (available from Invitrogen, Carlsbad, CA) that contain a selectable marker such as a gene that confers G418 resistance (which facilitates the selection of stably transfected cell lines) and the human cytomegalovirus (CMV) enhancer-promoter sequences. Additionally, suitable for expression in primate or canine cell lines is the pCEP4 vector (Invitrogen), which contains an Epstein Barr virus (EBV) origin of replication, facilitating the maintenance of 20 plasmid as a multicopy extrachromosomal element. Another expression vector is the pEF-BOS plasmid containing the promoter of polypeptide Elongation Factor 1 α , which stimulates efficiently transcription *in vitro*. The plasmid is described by Mishizuma and Nagata (*Nuc. Acids Res.* 18:5322, 1990), and its use in transfection experiments is disclosed by, for example, Demoulin (*Mol. Cell. Biol.* 16:4710-4716, 1996). Still another preferred expression 25 vector is an adenovirus, described by Stratford-Perricaudet, which is defective for E1 and E3 proteins (*J. Clin. Invest.* 90:626-630, 1992).

In addition to the biological vectors, chemical/physical vectors may be used to deliver a cynomolgous PGP nucleic acid or polypeptide to a target cell and facilitate uptake thereby. As used herein, a "chemical/physical vector" refers to a natural or synthetic molecule, other 30 than those derived from bacteriological or viral sources, capable of delivering the isolated cynomolgous PGP nucleic acid or polypeptide to a cell.

A preferred chemical/physical vector of the invention is a colloidal dispersion system.

Colloidal dispersion systems include lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system of the invention is a liposome. Liposomes are artificial membrane vesicles which are useful as a delivery vector *in vivo* or *in vitro*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2 - 4.0 μ can encapsulate large macromolecules. RNA, DNA, and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al., *Trends Biochem. Sci.*, v. 6, p. 77 (1981)). In order for a liposome to be an efficient nucleic acid transfer vector, one or more of the following characteristics should be present: (1) encapsulation of the nucleic acid of interest at high efficiency with retention of biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information.

Liposomes may be targeted to a particular tissue by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein. Ligands which may be useful for targeting a liposome to a particular cell will depend on the particular cell or tissue type. Additionally when the vector encapsulates a nucleic acid, the vector may be coupled to a nuclear targeting peptide, which will direct the cynomologous PGP nucleic acid to the nucleus of the host cell.

Liposomes are commercially available from Gibco BRL, for example, as LIPOFECTINTM and LIPOFECTACETM, which are formed of cationic lipids such as N-[1-(2,3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in the art and have been described in many publications.

Other exemplary compositions that can be used to facilitate uptake by a target cell of the cynomologous PGP nucleic acids include calcium phosphate and other chemical mediators of intracellular transport, microinjection compositions, electroporation and homologous recombination compositions (e.g., for integrating a cynomologous PGP nucleic acid into a preselected location within a target cell chromosome).

The invention also embraces so-called expression kits, which allow the artisan to prepare a desired expression vector or vectors. Such expression kits include at least separate portions of the previously discussed coding sequences. Other components may be added, as desired, as long as the previously mentioned sequences, which are required, are included.

It will also be recognized that the invention embraces the use of the cynomolgous PGP cDNA sequences in expression vectors, as well as to transfect host cells and cell lines, be these prokaryotic (e.g., *E. coli*), or eukaryotic (e.g., COS cells, yeast expression systems and recombinant baculovirus expression in insect cells). Especially useful are mammalian 5 cells such as human, pig, goat, primate, etc. They may be of a wide variety of tissue types, and include primary cells and cell lines. Specific examples include intestinal cells and liver cells. The expression vectors require that the pertinent sequence, i.e., those nucleic acids described *supra*, be operably linked to a promoter.

The invention also provides isolated cynomolgous PGP polypeptides which include 10 the amino acid sequences of SEQ ID NO:2 and SEQ ID NO:4, and fragments thereof, encoded by the cynomolgous PGP nucleic acids described above. Cynomolgous PGP polypeptides also embrace alleles, functionally equivalent variants and analogs (those non- allelic polypeptides which vary in amino acid sequence from the disclosed cynomolgous PGP polypeptides by 1, 2, 3, 4, 5, or more amino acids) provided that such polypeptides 15 retain cynomolgous PGP activity. Non-functional variants also are embraced by the invention; these are useful as antagonists of transporter function, as negative controls in assays, and the like. Such alleles, variants, analogs and fragments are useful, for example, alone or as fusion proteins for a variety of purposes including as a component of assays.

Fragments of a polypeptide preferably are those fragments which retain a distinct 20 functional capability of the cynomolgous PGP polypeptide, in particular as a transporter of various molecules. Other functional capabilities which can be retained in a fragment of a cynomolgous PGP polypeptide include interaction with antibodies and interaction with other polypeptides (such as would be found in a protein complex). Those skilled in the art are well versed in methods for selecting fragments which retain a functional capability of the 25 cynomolgous PGP. Confirmation of the functional capability of the fragment can be carried out by synthesis of the fragment and testing of the capability according to standard methods. For example, to test the transporter activity of a cynomolgous PGP fragment, one inserts or expresses the fragment in a cell in which molecular transport can be measured. Such methods, which are standard in the art, are described further herein.

30 The invention embraces variants of the cynomolgous PGP polypeptides described above. As used herein, a "variant" of a cynomolgous PGP polypeptide is a polypeptide which contains one or more modifications to the primary amino acid sequence of a

cynomologous PGP polypeptide. Modifications which create a cynomologous PGP variant can be made to a cynomologous PGP polypeptide for a variety of reasons, including 1) to reduce or eliminate an activity of a cynomologous PGP polypeptide, such as transport; 2) to enhance a property of a cynomologous PGP polypeptide, such as protein stability in an expression system or the stability of protein-protein binding; 3) to provide a novel activity or property to a cynomologous PGP polypeptide, such as addition of an antigenic epitope or addition of a detectable moiety; or 4) to establish that an amino acid substitution does or does not affect molecular transport activity. Modifications to a cynomologous PGP polypeptide are typically made to the nucleic acid which encodes the cynomologous PGP polypeptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids or non-amino acid moieties. Alternatively, modifications can be made directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety, such as biotin, addition of a fatty acid, and the like. Modifications also embrace fusion proteins comprising all or part of the cynomologous PGP amino acid sequence. One of skill in the art will be familiar with methods for predicting the effect on protein conformation of a change in protein sequence, and can thus "design" a variant cynomologous PGP according to known methods. One example of such a method is described by Dahiyat and Mayo in *Science* 278:82-87, 1997, whereby proteins can be designed *de novo*. The method can be applied to a known protein to vary only a portion of the polypeptide sequence. By applying the computational methods of Dahiyat and Mayo, specific variants of a cynomologous PGP polypeptide can be proposed and tested to determine whether the variant retains a desired conformation.

Variants include cynomologous PGP polypeptides which are modified specifically to alter a feature of the polypeptide unrelated to its physiological activity. For example, cysteine residues can be substituted or deleted to prevent unwanted disulfide linkages. Similarly, certain amino acids can be changed to enhance expression of a cynomologous PGP polypeptide by eliminating proteolysis by proteases in an expression system (e.g., dibasic amino acid residues in yeast expression systems in which KEX2 protease activity is present).

Mutations of a nucleic acid which encode a cynomologous PGP polypeptide preferably preserve the amino acid reading frame of the coding sequence, and preferably do not create regions in the nucleic acid which are likely to hybridize to form secondary structures, such as hairpins or loops, which can be deleterious to expression of the variant

polypeptide.

Mutations can be made by selecting an amino acid substitution, or by random mutagenesis of a selected site in a nucleic acid which encodes the polypeptide. Variant polypeptides are then expressed and tested for one or more activities to determine which 5 mutation provides a variant polypeptide with a desired property. Further mutations can be made to variants (or to non-variant cynomolgous PGP polypeptides) which are silent as to the amino acid sequence of the polypeptide, but which provide preferred codons for translation in a particular host. The preferred codons for translation of a nucleic acid in, e.g., *E. coli*, are well known to those of ordinary skill in the art. Still other mutations can be made 10 to the noncoding sequences of a cynomolgous PGP gene or cDNA clone to enhance expression of the polypeptide.

The activity of variants of cynomolgous PGP polypeptides can be tested by cloning the gene encoding the variant cynomolgous PGP polypeptide into a bacterial or mammalian expression vector, introducing the vector into an appropriate host cell, expressing the variant 15 cynomolgous PGP polypeptide, and testing for a functional capability of the cynomolgous PGP polypeptides as disclosed herein. For example, the variant cynomolgous PGP polypeptide can be tested for ability to provide molecular transport (e.g., efflux), as set forth below in the examples. Preparation of other variant polypeptides may favor testing of other activities, as will be known to one of ordinary skill in the art.

20 The skilled artisan will also realize that conservative amino acid substitutions may be made in cynomolgous PGP polypeptides to provide functionally equivalent variants of the foregoing polypeptides, i.e., variants which retain the functional capabilities of the cynomolgous PGP polypeptides. As used herein, a "conservative amino acid substitution" refers to an amino acid substitution which does not alter the relative charge or size 25 characteristics of the polypeptide in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. 30 Exemplary functionally equivalent variants of the cynomolgous PGP polypeptides include conservative amino acid substitutions of SEQ ID NO:2 or SEQ ID NO:4. Conservative

substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

Conservative amino-acid substitutions in the amino acid sequence of cynomologous PGP polypeptide to produce functionally equivalent variants of cynomologous PGP typically are made by alteration of the nucleic acid sequence encoding cynomologous PGP polypeptides (e.g., SEQ ID NOs:1 or 3). Such substitutions can be made by a variety of methods known to one of ordinary skill in the art. For example, amino acid substitutions may be made by PCR-directed mutation, site-directed mutagenesis according to the method of Kunkel (Kunkel, *Proc. Nat. Acad. Sci. U.S.A.* 82: 488-492, 1985), or by chemical synthesis of a gene encoding a cynomologous PGP polypeptide. The activity of functionally equivalent fragments of cynomologous PGP polypeptides can be tested by cloning the gene encoding the altered cynomologous PGP polypeptide into a bacterial or mammalian expression vector, introducing the vector into an appropriate host cell, expressing the altered cynomologous PGP polypeptide, and testing for the ability of the cynomologous PGP polypeptide to mediate transmembrane transport of compounds. Peptides which are chemically synthesized can be tested directly for function.

A variety of methodologies well-known to the skilled practitioner can be utilized to obtain isolated cynomologous PGP molecules. The polypeptide may be purified from cells which naturally produce the polypeptide by chromatographic means or immunological recognition. Alternatively, an expression vector may be introduced into cells to cause production of the polypeptide. In another method, mRNA transcripts may be microinjected or otherwise introduced into cells to cause production of the encoded polypeptide. Translation of mRNA in cell-free extracts such as the reticulocyte lysate system also may be used to produce polypeptide. Those skilled in the art also can readily follow known methods for isolating cynomologous PGP polypeptides. These include, but are not limited to, immunochromatography, HPLC, size-exclusion chromatography, ion-exchange chromatography and immune-affinity chromatography.

The invention as described herein has a number of uses, some of which are described elsewhere herein. For example, the invention permits isolation of the cynomologous PGP polypeptide molecules by e.g., expression of a recombinant nucleic acid to produce large quantities of polypeptide which may be isolated using standard protocols. As another

example, the isolation of the cynomolgous PGP gene makes it possible for cynomolgous PGP to be used in methods for assaying of molecular transport, such as drug bioavailability studies. These methods involve determining transport of a drug by a first species' PGP (e.g., cynomolgous, dog) in comparison to transport of the drug by other species' PGP (e.g. 5 human) as a method for determining or predicting the bioavailability of the drug. Thus the results of whole animal studies on the metabolism of a drug can be evaluated in view of the relative rates or amounts of P-glycoprotein transport of the drug. For example, if a drug administered to a dog has good oral bioavailability and low transport by dog PGP, one can predict that the oral bioavailability of the drug in humans will be good if the transport by 10 human PGP is also low. Conversely, if the transport of the drug by human PGP is high, then the bioavailability of the drug would be predicted to be low.

The invention also embraces agents which bind selectively to the cynomolgous PGP nucleic acid molecules or polypeptides as well as agents which bind to variants and fragments 15 of the polypeptides and nucleic acids as described herein. The agents include polypeptides which bind to cynomolgous PGP, and antisense nucleic acids, both of which are described in greater detail below. The agents can inhibit or increase cynomolgous PGP activity 20 (antagonists and agonists, respectively).

Some of the agents are inhibitors. A cynomolgous PGP inhibitor is an agent that inhibits cynomolgous PGP mediated transport of molecules across a cell membrane. Efflux 25 assays can be performed to screen and/or determine whether a cynomolgous PGP inhibitor has the ability to inhibit cynomolgous PGP activity, and whether the inhibition is selective. An exemplary assay of efflux is described below in the Examples.

In one embodiment the cynomolgous PGP inhibitor is an antisense oligonucleotide that selectively binds to a cynomolgous PGP nucleic acid molecule, to reduce the expression 25 of cynomolgous PGP (or other species' PGPs) in a cell. This is desirable in virtually any medical condition wherein a reduction of PGP transporter activity is desirable, e.g., to increase retention of cytotoxic agents in a cell.

As used herein, the term "antisense oligonucleotide" or "antisense" describes an oligonucleotide that is an oligoribonucleotide, oligodeoxyribonucleotide, modified 30 oligoribonucleotide, or modified oligodeoxyribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to an mRNA transcript of that gene and, thereby, inhibits the transcription of that gene and/or the translation of that

mRNA. The antisense molecules are designed so as to interfere with transcription or translation of a target gene upon hybridization with the target gene or transcript. Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected,

5 including the sequence of the target and the particular bases which comprise that sequence. It is preferred that the antisense oligonucleotide be constructed and arranged so as to bind selectively with the target under physiological conditions, i.e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological conditions. Based upon SEQ ID NOs:1 or 3, or upon allelic or homologous genomic and/or

10 cDNA sequences, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance with the present invention. In order to be sufficiently selective and potent for inhibition, such antisense oligonucleotides should comprise at least 10 and, more preferably, at least 15 consecutive bases which are complementary to the target, although in certain cases modified oligonucleotides as short as 7

15 bases in length have been used successfully as antisense oligonucleotides (Wagner et al., *Nature Biotechnol.* 14:840-844, 1996). Most preferably, the antisense oligonucleotides comprise a complementary sequence of 20-30 bases. Although oligonucleotides may be chosen which are antisense to any region of the gene or mRNA transcripts, in preferred embodiments the antisense oligonucleotides correspond to N-terminal or 5' upstream sites

20 such as translation initiation, transcription initiation or promoter sites. In addition, 3'- untranslated regions may be targeted. Targeting to mRNA splicing sites has also been used in the art but may be less preferred if alternative mRNA splicing occurs. In addition, the antisense is targeted, preferably, to sites in which mRNA secondary structure is not expected (see, e.g., Sainio et al., *Cell Mol. Neurobiol.* 14(5):439-457, 1994) and at which polypeptides

25 are not expected to bind. Thus, the present invention also provides for antisense oligonucleotides which are complementary to allelic or homologous cDNAs and genomic DNAs corresponding to cynomolgous PGP nucleic acid containing SEQ ID NOs:1 or 3.

In one set of embodiments, the antisense oligonucleotides of the invention may be composed of "natural" deoxyribonucleotides, ribonucleotides, or any combination thereof.

30 That is, the 5' end of one native nucleotide and the 3' end of another native nucleotide may be covalently linked, as in natural systems, via a phosphodiester internucleoside linkage. These oligonucleotides may be prepared by art recognized methods which may be carried out

manually or by an automated synthesizer. They also may be produced recombinantly by vectors.

In preferred embodiments, however, the antisense oligonucleotides of the invention also may include "modified" oligonucleotides. That is, the oligonucleotides may be modified 5 in a number of ways which do not prevent them from hybridizing to their target but which enhance their stability or targeting or which otherwise enhance their therapeutic effectiveness.

The term "modified oligonucleotide" as used herein describes an oligonucleotide in which (1) at least two of its nucleotides are covalently linked via a synthetic internucleoside 10 linkage (i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not normally associated with nucleic acids has been covalently attached to the oligonucleotide. Preferred synthetic internucleoside linkages are phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, 15 carbamates, carbonates, phosphate triesters, acetamides, carboxymethyl esters and peptides.

The term "modified oligonucleotide" also encompasses oligonucleotides with a covalently modified base and/or sugar. For example, modified oligonucleotides include oligonucleotides having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a 20 phosphate group at the 5' position. Thus modified oligonucleotides may include a 2'-O-alkylated ribose group. In addition, modified oligonucleotides may include sugars such as arabinose instead of ribose. The present invention, thus, contemplates pharmaceutical preparations containing modified antisense molecules that are complementary to and 25 hybridizable with, under physiological conditions, nucleic acids encoding cynomolgous PGP polypeptides, together with pharmaceutically acceptable carriers.

Agents which bind cynomolgous PGP also include binding peptides and other molecules which bind to the cynomolgous PGP polypeptide and complexes containing the cynomolgous PGP polypeptide. When the binding molecules are inhibitors, the molecules bind to and inhibit the activity of cynomolgous PGP. To determine whether a 30 cynomolgous PGP binding agent binds to cynomolgous PGP any known binding assay may be employed. For example, the binding agent may be immobilized on a surface and then contacted with a labeled cynomolgous PGP polypeptide. The amount of cynomolgous PGP

which interacts with the cynomolgous PGP binding agent or the amount which does not bind to the cynomolgous PGP binding agent may then be quantitated to determine whether the cynomolgous PGP binding agent binds to cynomolgous PGP.

The cynomolgous PGP binding agents include molecules of numerous size and type
5 that bind selectively or preferentially to cynomolgous PGP polypeptides, and complexes of both cynomolgous PGP polypeptides and their binding partners. These molecules may be derived from a variety of sources. For example, cynomolgous PGP binding agents can be provided by screening degenerate peptide libraries which can be readily prepared in solution, in immobilized form or as phage display libraries. Combinatorial libraries also can be
10 synthesized of peptides containing one or more amino acids. Libraries further can be synthesized of peptoids and non-peptide synthetic moieties.

Phage display can be particularly effective in identifying binding peptides useful according to the invention. Briefly, one prepares a phage library (using e.g. m13, fd, or lambda phage), displaying inserts from 4 to about 80 amino acid residues using conventional
15 procedures. The inserts may represent, for example, a completely degenerate or biased array. One then can select phage-bearing inserts which bind to the cynomolgous PGP polypeptide. This process can be repeated through several cycles of reselection of phage that bind to the cynomolgous PGP polypeptide. Repeated rounds lead to enrichment of phage bearing particular sequences. DNA sequence analysis can be conducted to identify the sequences of
20 the expressed polypeptides. The minimal linear portion of the sequence that binds to the cynomolgous PGP polypeptide can be determined. One can repeat the procedure using a biased library containing inserts containing part or all of the minimal linear portion plus one or more additional degenerate residues upstream or downstream thereof. Yeast two-hybrid screening methods also may be used to identify polypeptides that bind to the cynomolgous
25 PGP polypeptides. Thus, the cynomolgous PGP polypeptides of the invention, or a fragment thereof, can be used to screen peptide libraries, including phage display libraries, to identify and select peptide binding partners of the cynomolgous PGP polypeptides of the invention. Such molecules can be used, as described, for screening assays, for purification protocols, for interfering directly with the functioning of cynomolgous PGP and for other purposes that
30 will be apparent to those of ordinary skill in the art.

Therefore the invention generally provides efficient methods of identifying pharmacological agents or lead compounds for agents useful in the treatment of conditions

associated with aberrant PGP activity and the compounds and agents so identified. Generally, the screening methods involve assaying for compounds which inhibit or enhance transport of molecules through cynomolgous PGP. Such methods are adaptable to automated, high throughput screening of compounds. Examples of such methods are described in US patent
5 5,429,921.

A variety of assays for pharmacological agents are provided, including, labeled *in vitro* protein binding assays, efflux assays using detectable molecules, etc. For example, protein binding screens are used to rapidly examine the binding of candidate pharmacological agents to a cynomolgous PGP. The candidate pharmacological agents can be derived from,
10 for example, combinatorial peptide libraries. Convenient reagents for such assays are known in the art. An exemplary cell-based assay of efflux involves contacting a cell having a cynomolgous PGP with a candidate pharmacological agent under conditions whereby the efflux of a detectably labeled molecule can occur. Specific conditions are well known in the art and are described , for example, in Sharom et al., *Biochem. Pharmacol.* 58:571-586, 1999,
15 and references cited therein. A reduction in the efflux in the presence of the candidate pharmacological agent indicates that the candidate pharmacological agent reduces the efflux activity of cynomolgous PGP. An increase in the efflux in the presence of the candidate pharmacological agent indicates that the candidate pharmacological agent increases the efflux activity of cynomolgous PGP.

Cynomolgous PGP used in the methods of the invention can be added to an assay mixture as an isolated polypeptide (where binding of a candidate pharmaceutical agent is to be measured) or as a cell or other membrane-encapsulated space which includes a cynomolgous PGP polypeptide. In the latter assay configuration, the cell or other membrane-encapsulated space can contain the cynomolgous PGP as a preloaded polypeptide
20 or as a nucleic acid (e.g. a cell transfected with an expression vector containing a cynomolgous PGP). In the assays described herein, the cynomolgous PGP polypeptide can be produced recombinantly, or isolated from biological extracts, but preferably is synthesized *in vitro*. Cynomolgous PGP polypeptides encompass chimeric proteins comprising a fusion of a cynomolgous PGP polypeptide with another polypeptide, e.g., a polypeptide capable of
25 providing or enhancing protein-protein binding, or enhancing stability of the cynomolgous PGP polypeptide under assay conditions. A polypeptide fused to a cynomolgous PGP polypeptide or fragment thereof may also provide means of readily detecting the fusion
30

protein, e.g., by immunological recognition or by fluorescent labeling.

The assay mixture also comprises a candidate pharmacological agent. Typically, a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a different response to the various concentrations. Typically, one of these concentrations serves
5 as a negative control, i.e., at zero concentration of agent or at a concentration of agent below the limits of assay detection. Candidate agents encompass numerous chemical classes, although typically they are organic compounds. Preferably, the candidate pharmacological agents are small organic compounds, i.e., those having a molecular weight of more than 50 yet less than about 2500. Candidate agents comprise functional chemical groups necessary
10 for structural interactions with polypeptides, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups and more preferably at least three of the functional chemical groups. The candidate agents can comprise cyclic carbon or heterocyclic structure and/or aromatic or polyaromatic structures substituted with one or more of the above-identified functional groups. Candidate agents also
15 can be biomolecules such as peptides, saccharides, fatty acids, sterols, isoprenoids, purines, pyrimidines, derivatives or structural analogs of the above, or combinations thereof and the like. Where the agent is a nucleic acid, the agent typically is a DNA or RNA molecule, although modified nucleic acids having non-natural bonds or subunits are also contemplated.

Candidate agents are obtained from a wide variety of sources including libraries of
20 synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides, synthetic organic combinatorial libraries, phage display libraries of random peptides, and the like. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced.
25 Additionally, natural and synthetically produced libraries and compounds can be readily modified through conventional chemical, physical, and biochemical means. Further, known pharmacological agents may be subjected to directed or random chemical modifications such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs of the agents.

30 Therefore, a source of candidate agents are libraries of molecules based on known P-glycoprotein inhibitors, in which the structure of the inhibitor is changed at one or more positions of the molecule to contain more or fewer chemical moieties or different chemical

moieties. The structural changes made to the molecules in creating the libraries of analog inhibitors can be directed, random, or a combination of both directed and random substitutions and/or additions. One of ordinary skill in the art in the preparation of combinatorial libraries can readily prepare such libraries based on existing P-glycoprotein
5 inhibitors.

A variety of other reagents also can be included in the mixture. These include reagents such as salts, buffers, neutral proteins (e.g., albumin), detergents, etc. which may be used to facilitate optimal protein-protein and/or protein-nucleic acid binding. Such a reagent may also reduce non-specific or background interactions of the reaction components. Other
10 reagents that improve the efficiency of the assay such as protease inhibitors, nuclease inhibitors, antimicrobial agents, and the like may also be used.

The mixture of the foregoing assay materials is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the cynomolgous PGP mediates the efflux of a control amount of a compound such as a drug. For determining the binding of
15 a candidate pharmaceutical agent to a cynomolgous PGP, the mixture is incubated under conditions which permit binding. The order of addition of components, incubation temperature, time of incubation, and other parameters of the assay may be readily determined. Such experimentation merely involves optimization of the assay parameters, not the fundamental composition of the assay. Incubation temperatures typically are between 4°C
20 and 40°C. Incubation times preferably are minimized to facilitate rapid, high throughput screening, and typically are between 1 minute and 10 hours.

After incubation, the level of efflux or the level of specific binding between the cynomolgous PGP polypeptide and the candidate pharmaceutical agent is detected by any convenient method available to the user. For cell free binding type assays, a separation step is
25 often used to separate bound from unbound components. The separation step may be accomplished in a variety of ways. Conveniently, at least one of the components is immobilized on a solid substrate, from which the unbound components may be easily separated. The solid substrate can be made of a wide variety of materials and in a wide variety of shapes, e.g., microtiter plate, microbead, dipstick, resin particle, etc. The substrate
30 preferably is chosen to maximize signal to noise ratios, primarily to minimize background binding, as well as for ease of separation and cost.

Separation may be effected for example, by removing a bead or dipstick from a

reservoir, emptying or diluting a reservoir such as a microtiter plate well, rinsing a bead, particle, chromatographic column or filter with a wash solution or solvent. The separation step preferably includes multiple rinses or washes. For example, when the solid substrate is a microtiter plate, the wells may be washed several times with a washing solution, which

5 typically includes those components of the incubation mixture that do not participate in specific bindings such as salts, buffer, detergent, non-specific protein, etc. Where the solid substrate is a magnetic bead, the beads may be washed one or more times with a washing solution and isolated using a magnet.

Detection may be effected in any convenient way for cell-based assays such as a
10 transmembrane transport assay. The transport of a directly or indirectly detectable product, e.g., a fluorescent molecule such as calcein AM or rhodamine 123, is preferred. For cell free binding assays, one of the components usually comprises, or is coupled to, a detectable label. A wide variety of labels can be used, such as those that provide direct detection (e.g., radioactivity, luminescence, optical or electron density, etc.) or indirect detection (e.g.,
15 epitope tag such as the FLAG epitope, enzyme tag such as horseradish peroxidase, etc.). The label may be bound to a cynomologous PGP polypeptide or the candidate pharmacological agent.

A variety of methods may be used to detect the label, depending on the nature of the label and other assay components. For example, the label may be detected while bound to the
20 solid substrate or subsequent to separation from the solid substrate. Labels may be directly detected through optical or electron density, radioactive emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, streptavidin-biotin conjugates, etc. Methods for detecting the labels are well known in the art.

The cynomologous PGP binding agent may also be an antibody or a functionally active antibody fragment. Antibodies are well known to those of ordinary skill in the science of immunology. As used herein, the term "antibody" means not only intact antibody molecules but also fragments of antibody molecules retaining cynomologous PGP binding ability. Such fragments are also well known in the art and are regularly employed both *in vitro* and *in vivo*. In particular, as used herein, the term "antibody" means not only intact immunoglobulin molecules but also the well-known active fragments F(ab')₂, and Fab. F(ab')₂, and Fab fragments which lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody

(Wahl et al., *J. Nucl. Med.* 24:316-325 (1983)).

Monoclonal antibodies may be made by any of the methods known in the art utilizing cynomolgous PGP, or a fragment thereof, as an immunogen. Alternatively the antibody may be a polyclonal antibody specific for cynomolgous PGP which inhibits cynomolgous PGP
5 activity. The preparation and use of polyclonal antibodies is also known to one of ordinary skill in the art.

Significantly, as is well known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W.R. (1986) *The Experimental Foundations of Modern Immunology* Wiley & Sons, Inc., New York; Roitt, I. (1991) *Essential Immunology*, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an F(ab')₂ fragment, retains both of the antigen binding sites of an intact antibody. Similarly,
10 an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may
15 be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the
25 paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity.

30 In general, intact antibodies are said to contain "Fc" and "Fab" regions. The Fc regions are involved in complement activation and are not involved in antigen binding. An antibody from which the Fc' region has been enzymatically cleaved, or which has been

produced without the Fc' region, designated an "F(ab')₂" fragment, retains both of the antigen binding sites of the intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an "Fab" fragment, retains one of the antigen binding sites of the intact antibody. Fab'

5 fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain, denoted "Fd." The Fd fragments are the major determinants of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity). Isolated Fd fragments retain the ability to specifically bind to antigen epitopes.

10 The sequences of the antigen-binding Fab' portion of the anti-cynomologous PGP monoclonal antibodies identified as being useful according to the invention in the assays provided above, as well as the relevant FR and CDR regions, can be determined using amino acid sequencing methods that are routine in the art. It is well established that non-CDR regions of a mammalian antibody may be replaced with corresponding regions of non-specific or hetero-specific antibodies while retaining the epitope specificity of the original antibody.
15 This technique is useful for the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. Techniques to humanize antibodies are particularly useful when non-human animal (e.g., murine) antibodies which inhibit cynomologous PGP activity are identified. These non-human animal antibodies can be humanized for use in the treatment of
20 a human subject in the methods according to the invention. Examples of methods for humanizing a murine antibody are provided in U.S. patents 4,816,567, 5,225,539, 5,585,089, 5,693,762 and 5,859,205. Other antibodies, including fragments of intact antibodies with antigen-binding ability, are often referred to as "chimeric" antibodies.

25 Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab')₂, and Fab fragments of an anti-cynomologous PGP monoclonal antibody; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions of an anti-cynomologous PGP antibody have been replaced by homologous human or non-human sequences; chimeric F(ab')₂ fragment antibodies in which the FR and/or
30 CDR1 and/or CDR2 and/or light chain CDR3 regions of an anti-cynomologous PGP antibody have been replaced by homologous human or non-human sequences; and chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3

regions have been replaced by homologous human or non-human sequences.

According to the invention cynomolgous PGP inhibitors also include “dominant negative” polypeptides derived from SEQ ID NOs:2 or 4. A dominant negative polypeptide is an inactive variant of a polypeptide, which, by interacting with the cellular machinery, 5 displaces an active polypeptide from its interaction with the cellular machinery or competes with the active polypeptide, thereby reducing the effect of the active polypeptide. For example, a dominant negative receptor which binds a ligand but does not transmit a signal in response to binding of the ligand can reduce the biological effect of expression of the ligand.

The end result of the expression of a dominant negative cynomolgous PGP 10 polypeptide of the invention in a cell is a reduction in PGP activity such as molecular transport. One of ordinary skill in the art can assess the potential for a dominant negative variant of a cynomolgous PGP polypeptide, and using standard mutagenesis techniques to create one or more dominant negative variant polypeptides. For example, given the teachings contained herein of a cynomolgous PGP polypeptide, one of ordinary skill in the art can 15 modify the sequence of the cynomolgous PGP polypeptide by site-specific mutagenesis, scanning mutagenesis, partial gene deletion or truncation, and the like. See, e.g., U.S. Patent No. 5,580,723 and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. The skilled artisan then can test the population of mutagenized polypeptides for diminution in cynomolgous PGP activity and/or 20 for retention of such an activity. Other similar methods for creating and testing dominant negative variants of a cynomolgous PGP polypeptide will be apparent to one of ordinary skill in the art.

Each of the compositions of the invention is useful for a variety of therapeutic and 25 non-therapeutic purposes. For example, the cynomolgous PGP nucleic acids of the invention are useful as oligonucleotide probes. Such oligonucleotide probes can be used herein to identify genomic or cDNA library clones possessing an identical or substantially similar nucleic acid sequence. A suitable oligonucleotide or set of oligonucleotides, which is capable of hybridizing under stringent hybridization conditions to the desired sequence, a variant or fragment thereof, or an anti-sense complement of such an oligonucleotide or set of 30 oligonucleotides, can be synthesized by means well known in the art (see, for example, *Synthesis and Application of DNA and RNA*, S.A. Narang, ed., 1987, Academic Press, San Diego, CA) and employed as a probe to identify and isolate the desired sequence, variant or

fragment thereof by techniques known in the art. Techniques of nucleic acid hybridization and clone identification are disclosed by Sambrook, et al., *Molecular Cloning, A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Plainview, NY (1989). To facilitate the detection of a desired nucleic acid sequence, or variant or fragment thereof, whether for cloning purposes or for the mere detection of the presence of the sequence, the above-described probes may be labeled with a detectable group. Such a detectable group may be any material having a detectable physical or chemical property. Such materials have been well-developed in the field of nucleic acid hybridization and, in general, many labels useful in such methods can be applied to the present invention. Particularly useful are radioactive labels. Any radioactive label may be employed which provides for an adequate signal and has a sufficient half-life. If single stranded, the oligonucleotide may be radioactively labeled using kinase reactions. Alternatively, oligonucleotides are also useful as nucleic acid hybridization probes when labeled with a non-radioactive marker such as biotin, an enzyme or a fluorescent group. See, for example, Leary, J.J., et al., *Proc. Natl. Acad. Sci. (USA)* 80:4045 (1983); Renz, M. et al., *Nucl. Acids Res.* 12:3435 (1984); and Renz, M., *EMBO J.* 6:817 (1983).

Additionally, complements of the cynomolgous PGP nucleic acids can be useful as antisense oligonucleotides, e.g., by delivering the antisense oligonucleotide to an animal to induce a cynomolgous PGP “knockout” phenotype. The administration of antisense RNA probes to block gene expression is discussed in Lichtenstein, C., *Nature* 333:801-802 (1988).

Alternatively, the cynomolgous PGP nucleic acid of the invention can be used to prepare a non-human transgenic animal. A “transgenic animal” is an animal having cells that contain DNA which has been artificially inserted into a cell, which DNA becomes part of the genome of the animal which develops from that cell. Preferred transgenic animals are primates, mice, rats, cows, pigs, horses, goats, sheep, dogs and cats. Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Charles River (Wilmington, MA), Taconic (Germantown, NY), Harlan Sprague Dawley (Indianapolis, IN), etc. Transgenic animals having a particular property associated with a particular disease can be used to study the affects of a variety of drugs and treatment methods on the disease, and thus serve as genetic models for the study of a number of human diseases. The invention, therefore, contemplates the use of cynomolgous PGP knockout and transgenic animals as models for the study of disorders involving transport of molecules across

cell membranes. A variety of methods known to one of ordinary skill in the art are available for the production of transgenic animals associated with this invention.

Inactivation or replacement of the endogenous PGP/MDR1 gene can be achieved by a homologous recombination system using embryonic stem cells. The resultant transgenic non-
5 human mammals having a PGP^{-/-} knockout phenotype may be made transgenic for the cynomolgous PGP and used as a model for screening compounds as modulators (agonists or antagonists/inhibitors) of the cynomolgous PGP. In this manner, such therapeutic drugs can be identified.

Additionally, a normal or mutant version of cynomolgous PGP can be inserted into
10 the germ line to produce transgenic animals which constitutively or inducibly express the normal or mutant form of cynomolgous PGP. These animals are useful in studies to define the role and function of cynomolgous PGP in cells.

The compositions of the invention are also useful for therapeutic purposes.

Accordingly the invention encompasses a method for inhibiting cynomolgous PGP activity
15 in a mammalian cell. The invention further provides methods for reducing or increasing cynomolgous PGP activity in a cell. In one embodiment, the method involves contacting the mammalian cell with an amount of a cynomolgous PGP nucleic acid or polypeptide effective to inhibit molecular transport out of the mammalian cell. Such methods are useful
20 *in vitro* for the purpose of, for example, elucidating the mechanisms involved in drug resistance and reduced drug bioavailability.

The invention also encompasses a method for increasing PGP expression in a cell or subject. The amount of cynomolgous PGP can be increased in such cell or subject by contacting the cell with, or administering to the subject, a PGP nucleic acid or a PGP polypeptide of the invention to the subject in an amount effective to increase transmembrane
25 transport in the cell or the subject. An increase in PGP activity can be measured by the assays described herein, e.g., assays of transmembrane transport.

The preparations of the invention are administered in effective amounts. An effective amount is that amount of a pharmaceutical preparation that alone, or together with further doses, produces the desired response. Such amounts will depend, of course, on the particular
30 condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the

knowledge and expertise of the health practitioner. It is preferred generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons.

Generally, doses of active compounds would be from about 0.01 mg/kg per day to 1000 mg/kg per day. It is expected that doses ranging from 50-500 mg/kg will be suitable and in one or several administrations per day. Lower doses will result from other forms of administration, such as intravenous administration. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of compound, although fewer doses typically will be given when compounds are prepared as slow release or sustained release medications.

When administered, the pharmaceutical preparations of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptably compositions. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

The cynomolgous PGP inhibitors or cynomolgous PGP nucleic acids and polypeptides useful according to the invention may be combined, optionally, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions

also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

The pharmaceutical compositions may contain suitable buffering agents, including:

5 acetic acid in a salt; citric acid in a salt; and phosphoric acid in a salt.

The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular compound selected, the severity of the condition being
10 treated and the dosage required for therapeutic efficacy. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, interdermal, or parenteral routes. The term "parenteral" includes
15 subcutaneous, intravenous, intrathecal, intramuscular, or infusion. Intravenous or intramuscular routes are not particularly suitable for long-term therapy and prophylaxis.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes
20 one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active
25 compound. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the cynomologous PGP inhibitor or cynomologous PGP nucleic acids and polypeptides, which is preferably isotonic with the blood of the recipient. This aqueous
30 preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for

example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or 5 di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous, intravenous, intrathecal, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

Other delivery systems can include time-release, delayed release or sustained release 10 delivery systems such as the biological/chemical vectors is discussed above. Such systems can avoid repeated administrations of the active compound, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. Use of a long-term sustained release implant may be desirable. Long-term release, are used herein, means that the implant is constructed and 15 arranged to delivery therapeutic levels of the active ingredient for at least 30 days, and preferably 60 days. Long-term sustained release implants are well-known to those of ordinary skill in the art and include some of the release systems described above.

The invention will be more fully understood by reference to the following examples. These examples, however, are merely intended to illustrate the embodiments of the invention 20 and are not to be construed to limit the scope of the invention.

Examples

Example 1: Isolation of cynomolgous monkey P-glycoprotein

25 cDNA libraries were prepared using cynomolgous monkey (*Macaca fascicularis*) mRNA according to standard procedures. The libraries were screened for P-glycoprotein clones using a human P-glycoprotein DNA probe. Clones were isolated, purified and sequenced in accordance with standard procedures.

Preparation of Library

30 A custom Lambda ZAP II cDNA library from Cynomolgous monkey liver was prepared by Stratagene. This template was used to obtain clones 72/73 and 79/77 1500.

Anticipating that the monkey would show substantial homology to the human PGP,

initial primers were designed from the human PGP (Genbank Accession Number M14758). Primers were also made based on the Lambda ZAP II vector sequences. Later primers were designed based on the monkey sequence or a combination of the monkey/human sequences. All primers used are listed below.

5	Primer	Sequence	Source	SEQ NO	nucleotides
	PS070	ctg gac ttc ctc tca tga tgc tgg tgt	Human PGP	9	612-638 F
	PS072	gac agc tat tcg aag agt ggg cac aaa c	"	10	1550-1577 F
	PS073	ggc cat ggc acc aaa gac aac agc	"	11	3362-3385 R
	PS074	ttg gac aca gaa agt gaa gca gt	Cyno Monkey PGP	12	2105-2127 F (human seq)
	PS075	ctg agc atg gat cgg aaa ac	"	13	2798-2817 R (human seq)
	PS077	ttg taa tac gac tca cta tag ggc gaa t	T7 primer	14	"Based on Stratagene's seq.
	PS078	ctt ttc gag atg ggt aac tga agt gaa c	Cyno/human PGP	15	1613-1641 R
	PS079	aga agg tgc tgg gaa gat cgc tac tga a	"	16	3094-3121 F
	PS080	gcc taa agc cga aca cat	Cyno Monkey PGP	17	3498-3515 F
	PS081	cta tta agt ctg cat tct gga	"	18	4134-4154 R

All PCR reactions were done using a Perkin Elmer 9700 Thermocycler. PCR products were analyzed on an agarose gel, and promising bands were purified by the use of Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. These 10 bands were ligated into pCR 2.1 and transformed into INVaF using Invitrogen's TA Cloning protocol according to the manufacturer's instructions. White colonies were picked and analyzed by restriction digest. DNA was prepared from promising clones using the Promega Wizard Plus Miniprep DNA Purification System according to the manufacturer's instructions and sequenced with an ABI 377 sequencer.

Using primers ps072 and ps073, a ~1.8 kb fragment was obtained following 38 cycles of PCR (94°C for 5 m; followed by 38 cycles of 94° for 30 s, 63°C for 45 s, 72°C for 60 s; ending with 72°C for 7 m) using Klentaq. This was sequenced using m13F and m13R primers. Further sequencing primers, ps074 and ps075 were designed based on the sequence obtained. This resulted in a total of ~1.85 kb sequenced corresponding to human PGP 1553-3361.

Clone 79/77 C1500

Using primers ps079 and ps077, a ~1.5 kb fragment was obtained following 38 cycles of PCR (94°C for 5 m; followed by 38 cycles of 94°C for 30 s, 63°C for 45 s, 72°C for 60 s; ending with 72°C for 7 m) using Klentaq. This was sequenced using m13F and m13R as primers. Further sequencing primers, ps080 and ps081 were designed based on the sequence obtained. Sequence corresponding to the human pgp 3102 – 4525 was obtained, which included the stop codon for the cynomologous monkey PGP cDNA.

15

Clone 70/78C

Nucleobond RNA Maxi kit from Clontech was used according to the manufacturers to prepare total RNA from Cynomologous monkey liver. Single stranded cDNA was prepared from this RNA using the Superscript Kit from GIBCO BRL Life Technologies according to the manufacturer's instructions. This was used as a template for clones 70/78C. Using primers ps070 and ps078, a ~1.0 kb fragment was obtained from the liver cDNA following 38 cycles of PCR (94°C for 5 m; followed by 38 cycles of 94°C for 30 s, 65°C for 45 s, 72°C for 60 s; ending with 72°C for 7 m) using Klentaq. This was sequenced using m13F and m13R as primers. Sequence corresponding to the human pgp was obtained, which corresponded to human pgp 670 – 1638.

Clone 88/U 750

Nucleobond RNA Maxi kit from Clontech was used according to the manufacturer's instructions to prepare total RNA from Cynomologous monkey liver. Using the SMART Race cDNA Amplification Kit from Clontech, first strand cDNA was prepared from this RNA. The Universal Primer provided with this kit and the gene specific primer, ps088 were used with this template for 40 cycles of Touchdown PCR (94°C for 5 m; followed by 5 cycles

of 94°C for 30 s, 72°C for 120 s, 94°C for 30 s; 5 cycles of 94°C for 30 s, 70°C for 45s, 72°C for 120 s; 30 cycles of 94°C for 30 s, 68°C for 45 s, 72°C for 120 s; ending with 72°C for 7 m) using Advantage 2 Taq. A 750 nucleotide fragment was obtained and sequenced with m13F and m13R primers. This represented the 5' end of the cDNA including the start codon.
5 It showed good homology to the human sequence from 275 – 775 (the start codon for the human sequence being at 433).

Assembly of complete cDNA

Clone AB

10 Clone 88/U 750 was digested with NaeI / SacI. A 339 nucleotide fragment was isolated on a gel, and purified using the Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Clone 70/78C was digested with SacI / EcoRI, a 916 nucleotide fragment was isolated on a gel, and purified using the Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. These were ligated together into pUC
15 19 SmaI / EcoRI / SAP.

Clone CD

20 Clone 72/73 was digested with EcoRI / KpnI. A 1.6 kb fragment was isolated on a gel, and purified using the Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Clone 79/77 C1500 was digested with KpnI / DraI. A 1.1 kb fragment was isolated on a gel, and purified using the Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. These were ligated into pUC 19 EcoRI / SmaI / SAP.

Complete cDNA

Clone AB was digested with Hinc II / EcoRI. A 1.3 kb fragment was isolated on a gel, and purified using the Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

30 Clone CD was digested with EcoRI / EheI. The 5.2 kb fragment was isolated on a gel, and purified using the Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

These were ligated together, transformed into SCS-1 Competent Cells (Stratagene)

according to the manufacturer's instructions. Promising clones were identified by restriction digest. The identity of the final clone was confirmed by sequencing.

The nucleotide sequence of the cynomolgous P-glycoprotein is presented as SEQ ID NO:1. The coding sequence consists of nucleotides 100-3940, producing a polypeptide of 1280 amino acids (SEQ ID NO:2).

Sequencing of additional clones from libraries of individual cynomolgous monkeys indicated the presence of a polymorphism comprising an unexpected 9 base pair insert in the cDNA that is also present in the genomic sequence from the same individual monkey. The polymorphism resulted in an insertion of three amino acids after amino acid 92. The nucleotide sequence of this allelic variant is presented as SEQ ID NO:3. The coding sequence consists of nucleotides 100-3949, producing a polypeptide of 1283 amino acids (SEQ ID NO:4). The cynomolgous PGP cDNA (SEQ ID NO:3) cloned in plasmid pUC19 was deposited with ATCC (deposit number PTA-809).

15

Example 2: Activity of cynomolgous P-glycoprotein

Materials and Methods

Cynomolgous monkey PGP cDNA (SEQ ID NO:3) is introduced into a clonal population of LLC-PK1 cells in a vector that confers resistance to hygromycin B. LLC-PK1 cells are obtained from the American Type Culture Collections and are propagated in Medium 199 supplemented to 7% with fetal bovine serum. LLC-PK1 cells are recloned prior to transfection in order to assure homogeneity of the cell population. Briefly, cynomolgous monkey PGP cDNA is incorporated into the p222CMV vector. This vector is derived from the p220.2 episomal vector system based on the OriP sequences for Epstein Barr virus and the EBNA-1 gene product (Sugden et al., *Mol. Cell Biol.* 5:410-413, 1985; Yates et al., *Nature (Lond.)* 313: 812-815, 1985). The PGP cDNA is under the control of the cytomegalovirus (CMV) immediate early promoter. The vector confers resistance to hygromycin B. Cells (in 0.4 mL) and DNA (10 to 20 µg) were transfected by electroporation using a BTX Electro cell manipulator model 600 using a 2 mm gap cell, 100V, 2500 µF capacitance and 72 ohm resistance. After electroporation, the cells are plated in multiwell plates (48 well, Corning Costar) at 10% of confluence. One to two days after transfection hygromycin B is introduced at a final concentration of 400 to 600 µg/ml. Cells are refed every 2 to 4 days and are

propagated in 400 to 600 µg/ml hygromycin B for 6 to 8 days at which point the bulk of the wild type cells are detached. The hygromycin B is reduced to 100 µg/ml and maintained in this concentration of hygromycin B. After 14 to 18 days the wells are inspected and wells containing single colonies are trypsinized and scaled up to bulk cultures. Expression of PGP
5 is measured by the polarization of vinblastine (0.1 uM) transport in Transwells™.

LLC-PK1 cell based transport studies are conducted in 24 well Transwells™ (Corning Costar, Catalog number 3415). Transwells™ are prepared by the addition of 0.6 mL media to the basolateral space and 0.1 mL media to the apical space. Cells are seeded at 4×10^4 cells per insert (typically in 0.05 mL to 0.15 mL), refed with fresh media every 2 to 4 days and
10 used for transport studies 4 to 8 days post seeding. Transport assays are conducted in Hank's balanced saline (HBSS) buffered with 10 mM HEPES (pH 7 to 7.2). Cell monolayers are rinsed with HBSS prior to use in transport assays. Transport is measured under sink conditions in both the apical to basolateral (A to B) and basolateral to apical (B to A) directions. At least duplicate monolayers are used per determination. At the desired time
15 points, samples are withdrawn from the receiver chamber (apical or basolateral chambers). Quantitation of the amount of compound transported is by liquid scintillation counting (vinblastine) or HPLC with UV or mass spectrometric detection.

Cynomologous PGP cDNA is expressed in insect cells using a baculovirus vector. Membranes are prepared according to the method of (Sarkadi et al., *J. Biol. Chem.* 267:
20 4854-4858, 1992) and stored at -80°C until use. ATPase assays are conducted in 96 well microtiter plates. The assays are conducted using a modification of the methods of (Sarkadi et al., 1992 and Druekes et al., *Anal. Biochem.* 230: 173-177, 1995).

A detailed method for each well of a 96 well plate is contained below:

A 0.06 ml reaction mixture containing 40 µg membranes, 20 µM Verapamil (positive control)
25 or test drug, and 3-5 mM MgATP, in buffer containing 50 mM Tris-MES, 2 mM EGTA, 50 mM KCl, 2 mM dithiothreitol, and 5 mM sodium azide, is incubated at 37°C for 20 min. An identical reaction mixture containing 100 µM sodium orthovanadate is assayed in parallel. Orthovanadate inhibits PGP by trapping MgADP in the nucleotide binding site. Thus,
30 ATPase activity measured in the presence of orthovanadate represents non-PGP ATPase activity and can be subtracted from the activity generated without orthovanadate to yield vanadate-sensitive ATPase activity. The reaction is stopped by the addition of 30 µl of 10 % SDS + Antifoam A. Two additional reaction mixtures (+ and - orthovanadate) but without

MgATP, are also prepared and incubated with the others, and then supplemented with SDS and MgATP, to represent time = 0 min of reaction. The incubations are followed with addition of 200 μ l of 35 mM ammonium molybdate in 15 mM zinc acetate:10 % ascorbic acid (1:4) and incubated for an additional 20 min at 37°C. The liberation of inorganic phosphate is detected by its absorbance at 800 nm and quantitated by comparing the absorbance to a phosphate standard curve.

Ligand binding assays and assays for measuring inhibition of fluorescent dye uptake are performed as described by Sharom et al. (*Biochem. Pharmacol.* 58:571-586, 1999).

10 *I. Stable PGP Expression in LLC-PK1 Cells.*

Functional expression of cynomolgous monkey PGP is measured by the polarization of transport of vinblastine. Control cells typically demonstrate a B to A / A to B ratio of between 1 and 3. PGP transfected cells demonstrate a much higher ratio. The expression of cDNA-derived cynomolgous monkey is stable.

15

II. Activation of ATPase Activity in PGP Membranes.

The stimulation of ATPase assay provides a rapid measure of the concentration dependence of any interaction of a drug with PGP. The liberated inorganic phosphate is measured by a simple spectrophotometric assay performed in a microtiter plate format. The 20 testing of multiple drug concentrations allows estimation of the affinity of the drug for PGP and whether saturation of the response was observed.

III. Drug Transport Across Cell Monolayers.

The ATPase assay does not directly measure drug transport. In order to examine the 25 concordance between activation of ATPase and actual transport, the rates of transport of the drugs are measured in control LLC-PK1 and cynomolgous monkey PGP cell monolayers.

For each drug concentration, four measurements are made:

A:	A to B	Control cells
B:	B to A	Control cells
C:	A to B	PGP cells
D:	B to A	PGP cells

30 The polarization of transport is calculated in control cells (B/A) and PGP cells (D/C).

The intrinsic activity (IA) of PGP is calculated as the sum of the amount PGP facilitated B to A transport in PGP cells relative to control cells (D minus B) and the amount that PGP impeded A to B transport in PGP cells relative to control cells (A minus C). The intrinsic clearance of PGP is calculated from a plot of the concentration dependence data by either 5 calculating the slope of the line under non-saturating conditions or from the calculated apparent Km and Vmax values when saturation is observed. Intrinsic clearance is expressed as mL/m²/min.

The ATPase data provides useful concentration response data. For example, the apparent Km values for some compounds are in good agreement between the ATPase and 10 transport systems. However, other drugs activate ATPase activity but transport by PGP is not detectable. At the least, ATPase assay can identify a concentration range below which the response to transport by PGP was linear with respect to drug concentration. This should allow simplification of the experimental design for measuring the intrinsic clearance of PGP, an important consideration if large numbers of compounds are to be tested.

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IV. Bioavailability

Bioavailability studies are performed by performing one or more of the assays described above with two or more different PGP types. The different PGP types can be 20 different species (e.g., dog and human, cynomolgous monkey and human, dog and cynomolgous monkey, etc.) or can be different alleles of the same species. The results of these assays are compared to determine or estimate the bioavailability of a drug in individuals of the different species or in individuals that express different PGP alleles. The results of one determination also may be compared to a previously determined value of, e.g., ATPase or transport, as an historical control.

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Each of the foregoing patents, patent applications and references is hereby incorporated by reference. While the invention has been described with respect to certain embodiments, it should be appreciated that many modifications and changes may be made by those of ordinary skill in the art without departing from the spirit of the invention. It is 30 intended that such modification, changes and equivalents fall within the scope of the following claims.

What is claimed is: